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- The specification, claims, abstract and drawing as filed with the application on the filling date indicated above.

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Erhvervsministeriet

Taastrup 22 June 2000

Karin Schlichting
Head Clerk

NOVEL BUFFER

The present invention relates to the use of compounds of formulas (I)-(V) and/or salts thereof as defined herein as buffer components. The invention further relates to buffers comprising compounds of formulas (I)-(V) and/or salts thereof as defined herein. Furthermore, the invention relates to the use of such buffers in particular for zone electrophoresis and/or immunofixation. The invention also concerns kits for zone electrophoresis and/or immunofixation.

BACKGROUND OF THE INVENTION

The principle of immunofixation was described by Alfonzo and Wilson in 1964 (ref. 1). The method was later modified by Alper and Johnson in 1969 and used for identification of genetic protein variants (ref. 2). Immunofixation is a widely used diagnostic method. It is a rapid, important and useful tool for the examination and identification of various protein abnormalities in serum, urine, cerebrospinal and synovial fluids.

The immunofixation procedure can be used for the identification of any single protein band of an electrophoresis. The technique is a combination of zone electrophoresis followed by immunofixation using monospecific antibodies. In this way it is possible to separate and identify different proteins in a biological mixture according to their physicochemical properties and antigenic properties.

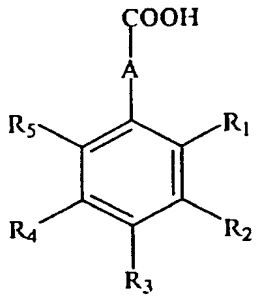
The immunofixation procedure is most frequently used for the detection of monoclonal immunoglobulins in serum and Bence Jones proteins in urine.

Usually, barbital buffers comprising barbituric acid and/or sodium barbiturate are used (ref. 3). In fact, this use is recommended as barbituric acid/sodium barbiturate provide a good separation of all protein bands. However, barbituric acid and sodium barbiturate are hazardous compounds which potentially cause irritation by contact with the skin, the eyes or the respiratory system, and which in extreme cases even may cause death. In more and more countries, the use of barbituric acid and sodium barbiturate in buffers is therefore prohibited.

Thus, buffer components which can replace barbituric acid and sodium barbiturate and which further possess the advantages of barbituric acid and/or sodium barbiturate are needed. The present invention provides such compounds which can replace barbituric acid and sodium barbiturate.

SUMMARY OF THE INVENTION

In a first aspect, the present invention relates to the use of a compound of the general formula (I)

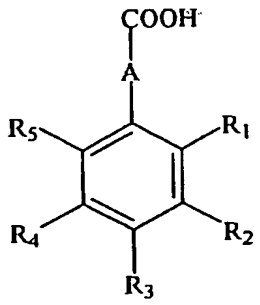


(I)

wherein R_1, R_2, R_3, R_4, and R_5 independently are selected from -H, -COOH, $-NO_2$, -OH, $-CH_3$, $-CF_3$, and $-CH_2CH_3$, and A is a bond or a group selected from -CH=CH-,
and/or a salt thereof

as a buffer component.

In another aspect, the present invention relates to such buffers comprising a compound of the general formula (I)



(I)

wherein R_1, R_2, R_3, R_4, and R_5 independently are selected from -H, -COOH, $-NO_2$, -OH, $-CH_3$, $-CF_3$, and $-CH_2CH_3$, and A is a bond or a group selected from -CH=CH-, and/or a salt thereof.

In a third aspect, the present invention relates to the use of the buffer as described herein for zone electrophoresis and/or immunofixation.

Furthermore, the present invention concerns kits for zone electrophoresis and/or immunofixation comprising a buffer as described herein.

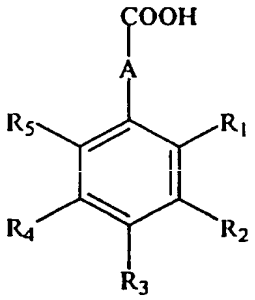
The present invention is described in detail in the following.

BRIEF DESCRIPTION OF THE FIGURE

Figure 1 shows an immunofixation gel obtained following the procedures described in Example 1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of a compound of the general formula (I)

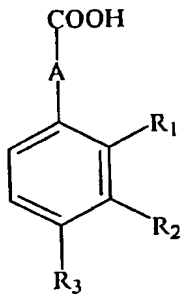


(I)

wherein R_1, R_2, R_3, R_4, and R_5 independently are selected from -H, -COOH, $-NO_2$, -OH, $-CH_3$, $-CF_3$, and $-CH_2CH_3$, and A is a bond or a group selected from -CH=CH-,
and/or a salt thereof

as a buffer component.

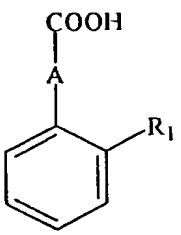
In one embodiment, a compound of the general formula (II) which is a compound of the general formula (I)



(II)

wherein R_1, R_2, and R_3 independently are selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined above, and/or a salt thereof, is used.

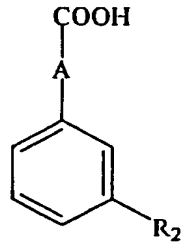
In another embodiment, a compound of the general formula (III) which is a compound of the general formula (I)



(III)

wherein R_1 is selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined above, and/or a salt thereof, is used. In particular, a compound of the formula (III), wherein A denotes a bond, may be used.

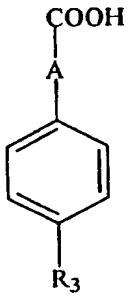
In a third embodiment, a compound of the general formula (IV) which is a compound of the general formula (I)



(IV)

wherein R_2 is selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined above, and/or a salt thereof, is used. In particular, a compound of the formula (IV), wherein A denotes a bond, may be used.

In a fourth embodiment, a compound of the general formula (V) which is a compound of the general formula (I)



(V)

wherein R_3 is selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined above, and/or a salt thereof, is used. In particular, a compound of the formula (V), wherein A denotes a bond, may be used.

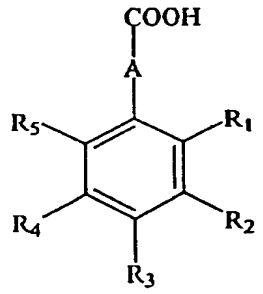
It is to be understood that when A denotes a bond, the -COOH group is bound directly to the aromatic ring.

In a particular embodiment, the compound of the general formula (I) is selected from benzoic acid, phthalic acid, terephthalic acid, o-toluic acid, m-toluic acid, p-toluic acid, cinnamic acid, o-nitrobenzoic acid, m-benzoic acid, p-nitrobenzoic acid, and salicylic acid, and/or salts thereof.

As indicated above, salts of the compounds may also be used. Suitable salt forms include sodium salts, potassium salts, calcium salts and/or magnesium salts.

In a special embodiment, benzoic acid or a salt thereof, in particular the sodium salt, is used as the buffer component.

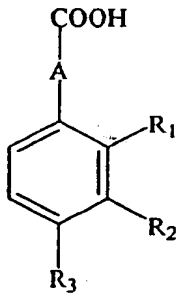
In another aspect, the present invention relates to buffers comprising a compound of the general formula (I)



(I)

wherein R_1, R_2, R_3, R_4, and R_5 independently are selected from -H, -COOH, $-NO_2$, -OH, $-CH_3$, $-CF_3$, and $-CH_2CH_3$, and A is a bond or a group selected from -CH=CH-,
and/or a salt thereof.

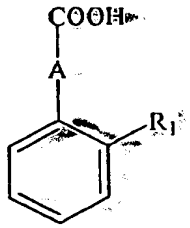
In one embodiment, the buffer of the invention comprises a compound of the general formula (II) which is a compound of the general formula (I)



(II)

wherein R_1, R_2, and R_3 independently are selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined above, and/or a salt thereof.

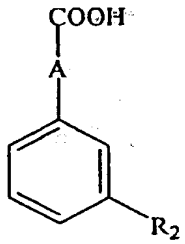
In another embodiment, the buffer comprises a compound of the general formula (III) which is a compound of the general formula (I)



(III)

wherein R_1 is selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined above, and/or a salt thereof. In particular, the compound may be a compound of formula (III) wherein A denotes a bond.

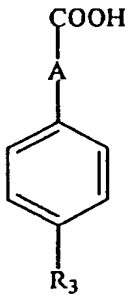
In a third embodiment, the buffer comprises a compound of the general formula (IV) which is a compound of the general formula (I)



(IV)

wherein R_2 is selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined above, and/or a salt thereof. In particular, the compound may be a compound of formula (IV) wherein A denotes a bond.

I a fourth embodiment, the buffer comprises a compound of the general formula (V) which is a compound of the general formula (I)



(V)

wherein R_3 is selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined above, and/or a salt thereof. In particular, the compound may be a compound of formula (V) wherein A denotes a bond.

In particular, the buffer of the invention may comprise a compound of the general formula (I) selected from benzoic acid, phthalic acid, terephthalic acid, o-toluic acid, m-toluic acid, p-toluic acid, cinnamic acid, o-nitrobenzoic acid, m-benzoic acid, p-nitrobenzoic acid, and salicylic acid, and/or salts thereof.

It is to be understood that the buffer of the invention may comprise one or more compounds as defined above and/or one or more salts of such compounds.

Salts of the compounds as defined above include sodium salts, potassium salts, calcium salts and/or magnesium salts.

In a special embodiment, the buffer comprises benzoic acid or a salt thereof, in particular the sodium salt.

The compounds as defined above are suitable as buffer components, in particular as gel buffers and compartment buffers. They are much less hazardous than the conventionally used barbiturates. Furthermore, no hazard labelling of the buffers comprising most of the compounds of formulas (I)-(V) is required. It has further been shown (cf. Example 1) that the buffers comprising the compounds defined above seem to provide shaper and more well-defined bands than the conventionally used barbiturate-containing buffers.

Furthermore, the buffer may contain one or more additional components such as buffering agents, preserving agents, colouring agents, salts, detergent and surfactants.

In one embodiment, the buffer further comprises Tris and/or Tricine and/or calcium lactate and/or sodium azide.

The buffer is suitable for use in zone electrophoresis, and/or immunofixation. The test samples are suitably serum, urine, cerebrospinal or synovial fluids.

In further aspect, the present invention relates to kits for zone electrophoresis and/or immunofixation, which kits comprise a buffer as defined above.

In one embodiment, the kit further comprises gels containing the buffer of the invention, staining solutions, antibodies, blotters, templates, fixation reagents, and/or immunoglobulins.

The invention is further illustrated by the following, non-limiting example.

EXAMPLES

EXAMPLE 1

Materials. Agarose gel, 10 plates. Ready-to-use. Each plate is 8.3×10.2 cm and contains on a transparent, flexible plastic backing, agarose gel in the buffer of the invention comprising sodium benzoate (i.e. sodium salt of benzoic acid) (1% gel, 99% buffer) preserved with sodium azide.

Concentrated buffer. 3×75 ml (13.33 × concentrated) buffer of the invention preserved with sodium azide. The content of each of the bottles of buffer is diluted prior to use to a total volume of 1000 ml with distilled water. The diluted buffer contains sodium benzoate (3.5 g/l), Tris (3.6 g/l), Tricine (0.6 g/l), calcium lactate (0.75 g/l), and sodium azide (0.04 g/l).

Concentrated staining solution. 75 ml (4 × concentrated). Amido Black. The staining solution is diluted prior to use to a total volume of 300 ml with distilled water. The concentration of Amido Black in the diluted solution is 5 g/l.

Test sample. Serum samples, preferably freshly drawn from fasting subjects. 33 samples were tested.

Sample template. 10 pieces.

Antibody template. 10 pieces.

Gel blotter. Pre-cut disposable, filter paper, 1 package, 40 sheets each.

Sample blotter. Pre-cut disposable, filter paper, 1 package, 10 sheets.

Drying blotter. Pre-cut disposable filter paper, 2 package, 20 sheets each.

Fixation reagents. Protein fixative solution 1.0 ml containing 7% sulphosalicylic acid and 5% acetic acid. Green dyed.

Rabbit anti-human IgG. Specific for γ-chains. Immunoglobulin fraction. 1.0 ml. Preserved with 15 mM sodium azide. Green dyed.

Rabbit anti-human IgA. Specific for α-chains. Immunoglobulin fraction. 1.0 ml. Preserved with 15 mM sodium azide. Green dyed.

Rabbit anti-human IgM. Specific for μ-chains. Immunoglobulin fraction. 1.0 ml. Preserved with 15 mM sodium azide. Green dyed.

Rabbit anti-human Kappa Light Chains. Immunoglobulin fraction. 1.0 ml. Preserved with 15 mM sodium azide. Green dyed.

Rabbit anti-human Lambda Light Chains. Immunoglobulin fraction. 1.0 ml. Preserved with 15 mM sodium azide.

Other reagents. Saline solution (0.9% NaCl). For dilution of the samples and washing of the gel. Destaining solution (acetic acid, 5%). Distilled or deionised water.

Equipment. Power supply 120 V constant. Electrophoresis apparatus for Agarose gels (DAKO Electrophoresis Apparatus Code No. S 2200). Pipettes (5 µl, 80 µl). Containers for washing, staining and destaining of agarose gels (DAKO Washing and Staining Accessory Kit Code No. S 2201). Glass plate (minimum 11×11 cm) plus a weight of approximately 1 kg for pressing the gel. Hair dryer or a drying oven (maximum 90°C).

Additional reagents. Rabbit anti-human IgD (DAKO Code No. A 0093), specific for δ-chains, immunoglobulin fraction, preserved with 15 mM sodium azide. Rabbit anti-human IgE (DAKO Code No. A 0094), specific for ε-chains, immunoglobulin fraction, preserved with 15 mM sodium azide. Rabbit anti-human Kappa Free Light Chains (DAKO Code No. A 0100), immunoglobulin fraction, preserved with 15 mM sodium azide. Rabbit anti-human Lambda Free Light Chains (DAKO Code No. A 0101), immunoglobulin fraction, preserved with 15 mM sodium azide.

Preparation of specimens. All serum specimens should preferably be diluted with saline solution just prior to use. For the reference pattern, serum should be diluted 1:4 (1 part serum + 3 parts saline solution). For the immunofixation patterns serum should be diluted 1:15 (1 part serum + 14 parts saline solution). For serum suspected of containing low levels of monoclonal immunoglobulins, a dilution of 1:4 is recommended. For serum specimens suspected of containing high levels of monoclonal immunoglobulin (>30 g/l), a dilution of 1:31 may be suitable.

For the detection of Bence Jones proteins in urine, the urine sample should be concentrated (e.g. by ultrafiltration) to a total protein concentration of at least 1 g/l. This concentrated urine is applied in all

slots. The light chain antibodies as described above will precipitate kappa or lambda chains whether they are free or still part of the immunoglobulin molecule. In order to determine if detected light chains are present as free light chains in the urine, special antibodies as described above against free Kappa and free Lambda light chains could be employed.

Assay procedure

Zone electrophoresis (separation of the proteins). All samples are prepared as described above. The agarose gel is removed from the foil package and placed on a level surface. Excess moisture is removed from the gel surface by gentle blotting with a Gel Blotter. The sample template is placed on the surface of the gel so that the slots are in alignment with the arrows located on the edges of the gel. 5 µl of the pre-diluted serum sample is applied across each slot. The 1:4 serum dilution is applied in the slot marked Ref., and the 1:15 serum dilution in the other 5 slots. The sample is allowed to diffuse into the gel for 5 minutes, and then the sample template is blotted gently with a Sample Blotter in order to remove excess sample liquid. The blotter is discarded, and the sample template is carefully removed and discarded.

Electrophoresis. The DAKO Electrophoresis Apparatus is filled with 300 ml diluted buffer (150 ml in each compartment). The gel is placed in the apparatus so as to form an arch (gel side down) in such as way that the (-) side of the gel dips into the cathode compartment (-), and that the (+) side of the gel dips into the anode compartment (+). The lid is placed on the apparatus and power supply is connected. The voltage is set to 120 V and the electrophoresis is continued for 25 minutes. Upon completion of the electrophoresis, the power supply is

REFERENCES

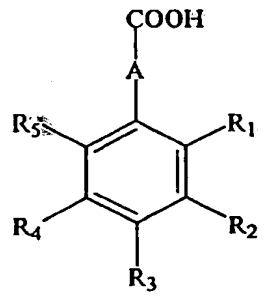
1. Alfonzo and Wilson, Clin. Chim. Acta 10, 114-122 (1964)

2. Alper CA and Johnson AM, Vox Sang. 17, 445-452 (1969)

3. Axelsen NH, Krøll J, and Weeke B, Scandinavian Journal of Immunology Vol. 2, Supplement No. 1, 25 (1973)

CLAIMS

1. Use of a compound of the general formula (I)

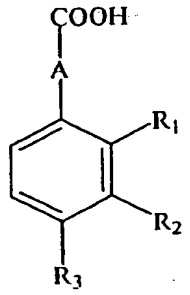


(I)

wherein R_1, R_2, R_3, R_4, and R_5 independently are selected from -H, -COOH, $-NO_2$, -OH, $-CH_3$, $-CF_3$, and $-CH_2CH_3$, and A is a bond or a group selected from -CH=CH-,
and/or a salt thereof

as a buffer component.

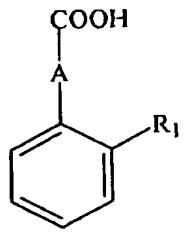
2. Use according to claim 1, wherein the compound of the general formula (I) is a compound of the general formula (II)



(II)

wherein R_1, R_2, and R_3 independently are selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined in claim 1,
and/or a salt thereof.

3. Use according to claim 1, wherein the compound of the general formula (I) is a compound of the general formula (III)

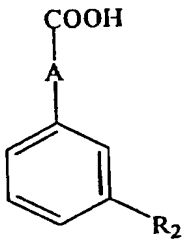


(III)

wherein R_1 is selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined in claim 1,
and/or a salt thereof.

4. Use according to claim 3, wherein A is a bond.

5. Use according to claim 1, wherein the compound of the general formula (I) is a compound of the general formula (IV)

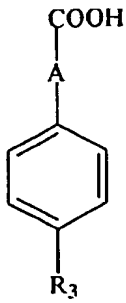


(IV)

wherein R_2 is selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined in claim 1,
and/or a salt thereof.

6. Use according to claim 5, wherein A is a bond.

7. Use according to claim 1, wherein the compound of the general formula (I) is a compound of the general formula (V)



(V)

wherein R_3 is selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined in claim 1,
and/or a salt thereof.

8. Use according to claim 7, wherein A is a bond.

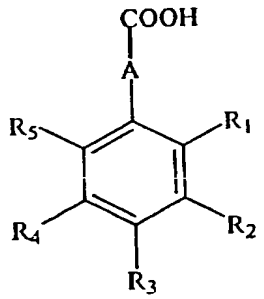
9. Use according to any one of claims 1-8, wherein the compound of the general formula (I) is selected from benzoic acid, phthalic acid, terephthalic acid, o-toluic acid, m-toluic acid, p-toluic acid, cinnamic acid, o-nitrobenzoic acid, m-benzoic acid, p-nitrobenzoic acid, and salicylic acid, and/or a salt thereof.

10. Use according to any one of claims 1-9, wherein the salt is a sodium salt, a potassium salt, a calcium salt and/or a magnesium salt.

11. Use according to any one of claims 1-10, wherein the buffer further comprises Tris and/or Tricine and/or calcium lactate and/or sodium azide.

12. Use according to any one of claims 1-11, wherein the compound is benzoic acid and/or a salt thereof, preferably the sodium salt.

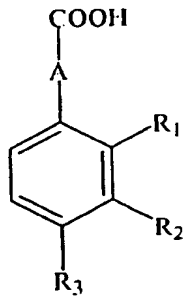
13. Buffer comprising a compound of the general formula (I)



(I)

wherein R_1, R_2, R_3, R_4, and R_5 independently are selected from -H, -COOH, $-NO_2$, -OH, $-CH_3$, $-CF_3$, and $-CH_2CH_3$, and A is a bond or a group selected from -CH=CH-,
and/or a salt thereof.

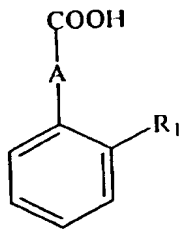
14. Buffer according to claim 13, wherein the compound of the general formula (I) is a compound of the general formula (II)



(II)

wherein R_1, R_2, and R_3 independently are selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined in claim 13,
and/or a salt thereof.

15. Buffer according to claim 13, wherein the compound of the general formula (I) is a compound of the general formula (III)

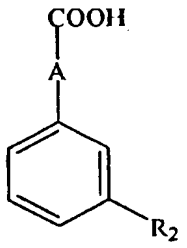


(III)

wherein R_1 is selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined in claim 13,
and/or a salt thereof.

16. Buffer according to claim 15, wherein A is a bond.

17. Buffer according to claim 13, wherein the compound of the general formula (I) is a compound of the general formula (IV)

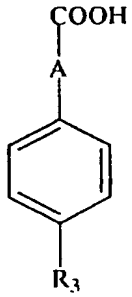


(IV)

wherein R_2 is selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined in claim 13,
and/or a salt thereof.

18. Buffer according to claim 17, wherein A is a bond.

19. Buffer according to claim 13, wherein the compound of the general formula (V)



(V)

wherein R_3 is selected from -H, -COOH, $-NO_2$, -OH, and $-CH_3$, and A is as defined in claim 13,

and/or a salt thereof.

20. Buffer according to claim 19, wherein A is a bond.

21. Buffer according to any one of claims 13-20, wherein the compound of the general formula (I) is selected from benzoic acid, phthalic acid, terephthalic acid, o-toluic acid, m-toluic acid, p-toluic acid, cinnamic acid, o-nitrobenzoic acid, m-benzoic acid, p-nitrobenzoic acid, and salicylic acid, and/or a salt thereof.

22. Buffer according to any one of claims 13-21, wherein the salt is a sodium salt, a potassium salt, a calcium salt and/or a magnesium salt.

23. Buffer according to any one of claims 13-22, wherein the buffer further comprises Tris and/or Tricine and/or calcium lactate and/or sodium azide.

24. Buffer according to any one of claims 13-23, wherein the compound is benzoic acid and/or a salt thereof, preferably the sodium salt.

25. Use of the buffer according to any one of claims 13-24 for zone electrophoresis and/or immunofixation.

26. Kit for zone electrophoresis and/or immunofixation comprising a buffer as defined in any one of claims 13-25.

27. Kit according to claim 26 further comprising gels containing the buffer as defined in any one of claims 13-25, staining solutions, antibodies, blotters, templates, fixation reagents, and/or immunoglobulins.

ABSTRACT

The use of compounds of formulas (I)-(V) and/or salts thereof as buffer components is disclosed. Furthermore, buffers comprising such compounds and/or salts are provided. The buffers may suitably be used for zone electrophoresis and/or immunofixation. Kits for zone electrophoresis and/or immunofixation are also provided.

Fig. 1